Characterization and angiogenic potential of xenogeneic bone grafting materials: Role of periodontal ligament cells

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Adequate revascularization is a prerequisite for successful healing of periodontal bone defects. This study characterized three different xenogeneic bone grafting materials: Gen-Os of equine and porcine origins, and anorganic Bio-Oss. We also investigated their angiogenic potential. All materials were composed of poorly crystalline calcium oxide phosphate, with Bio-Oss exhibiting a carbonated phase and larger particle size and both Gen-Os showing the presence of collagen. Both Gen-Os materials significantly enhanced vascular endothelial growth factor (VEGF) secretion by PDL cells. A significant increase in endothelial cell proliferation was observed in cultures with both Gen-Os conditioned media, but not with that of Bio-Oss. Finally, angiogenesis was stimulated by both Gen-Os conditioned media as demonstrated by an increased formation of capillary-like structures. Taken together, these findings indicate an enhanced angiogenic potential of both Gen-Os bone grafting materials when applied on PDL cells, most likely by increasing VEGF production.

Keywords: Characterization, Angiogenesis, VEGF, Bone substitute, Periodontal ligament cells

INTRODUCTION

Bone grafting materials are frequently applied in post-extraction ridge preservation and intrabony defect regeneration. These materials provide structural and biological support for bone regeneration and allow the migration of bone forming cells into the bone defect[1]. Successful bone regeneration is determined by the formation and migration of blood vessels from adjacent vital bone into the bone grafting material. Indeed, failure of rapid revascularization will impair the tissue regeneration process. Not only there will be a lack of oxygen and nutrients supply, but the circulation of cells required for bone tissue formation will be hampered as well[2-4].

To stimulate revascularization, researchers have explored the addition of exogenous angiogenic growth factors, like vascular endothelial growth factor (VEGF), into the bone defect site[5,6]. Although promising, there are several drawbacks with this approach. Due to the complexity of the *in vivo* environment and the short half-lives of growth factors, the natural spatio-temporal release profile of growth factors cannot be easily simulated by exogenous delivery. Often, supra-physiological doses are administered with possible adverse effects[5,7]. Furthermore, recombinant protein costs represent a significant financial burden for the patient[8].

Interestingly, it has been shown that various bone grafting materials, successfully used in clinic, have a high angiogenic potential. For instance, various *in vitro* studies showed increased VEGF and fibroblast growth factor (FGF-2) secretion by fibroblasts, after direct and indirect contact with 45S5 Bioglass® particles, which was associated with increased endothelial cell proliferation and formation of a tubular network[9-12]. A clinical study demonstrated increased angiogenesis following the application of a nanocrystalline hydroxyapatite paste in post-extractive sockets[13].

This suggests that the use of bone grafting materials with an angiogenic potential for treatment of periodontal bone defects can improve the clinical outcome. This study characterized three commonly used xenogeneic bone grafting materials in implantology and periodontics: Gen-Os cortico-cancellous collagenated bone of equine and porcine origins and Bio-Oss cancellous anorganic bovine bone, and evaluated their angiogenic potential. The rational beyond this study is that, in cases such as alveolar bone resorption after tooth loss and osseous periodontal lesions, the bone grafting material is not only in intimate contact with bone tissue but also with the periodontal ligament (Fig. 1). It is hypothesized that the material may influence periodontal ligament (PDL) cell angiogenic activity, which is a prerequisite for successful bone regeneration. In clinic, some PDL cells are subjected to the bone grafting material directly. However, the major part of PDL cells are subjected to extracts of these materials. This *in vitro* work simulates this clinical situation by subjecting PDL cells to extracts of the three bone grafting materials. This is followed by measurement of VEGF secretion, the most essential growth factor in

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the angiogenic process[14]. The influence on angiogenesis is further studied by evaluating endothelial cell proliferation and the formation of closed capillary-like structures reflecting the angiogenic capacity *in vivo*.

MATERIALS AND METHODS

Three different xenogeneic bone-grafting materials were assessed (Table 1 and Fig. 2):

- Bio-Oss cancellous anorganic bovine bone grafting material (Geistlich Pharma, Wolhusen, CH).
- Gen-Os cortico-cancellous collagenated bone grafting material of equine origin (OsteoBiol®, Tecnoss®, Giaveno, Italy).
- Gen-Os cortico-cancellous collagenated bone grafting material of porcine origin (OsteoBiol®, Tecnoss®).

Material characterization

The three bone grafting materials were characterized by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), X-ray diffraction (XRD) analyses and Fourier transform infrared (FT-IR) spectroscopy.

Powders sprinkled on carbon tape on an aluminum stub were viewed under the scanning electron microscope (Zeiss MERLIN Field Emission SEM, Carl Zeiss NTS, Oberkochen, Germany) in secondary electron mode at various magnifications. Energy dispersive spectroscopic (EDS) analysis was also performed. Phase analysis was performed by X-ray diffraction. The diffractometer (Bruker D8 Advance, Bruker, Billerica, MA, USA) used Cu Kα radiation at 40 mA and 45 kV and the detector was rotated between 10–60° with a step of 0.02°2θ and a step time of 0.6 degrees/min. The sample holder was spun at 15 rpm. Phase identification was accomplished using search-match software utilizing ICDD database
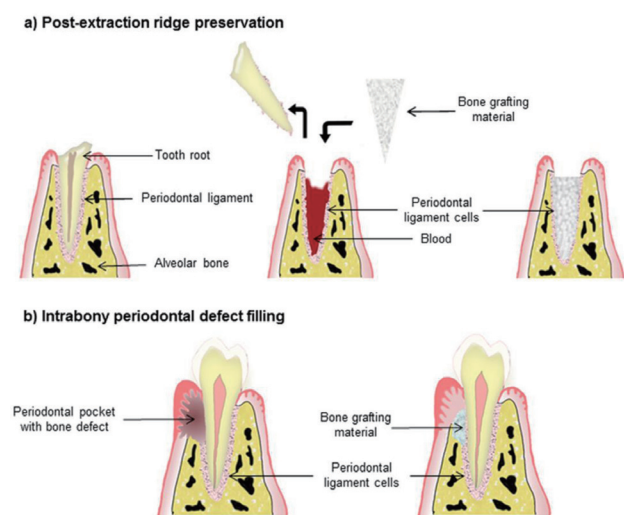

Fig. 1 Overview of bone grafting materials applications in periodontal pathologies and their interaction with the periodontal ligament.
(a) Post-extraction ridge preservation. After extraction of the tooth, bone grafting granules are added to the socket where they are in direct contact with PDL cells, blood and alveolar bone. The bone grafting material will reduce bone crest resorption at the socket walls. The PDL cells, being in close contact with the material, can influence angiogenesis and the bone regeneration process. (b) Intrabony periodontal defect filling. At the injured site of the periodontal pocket, the inflamed tissue is completely removed and a bone grafting material is placed inside the bone defect. The remaining PDL cells on the root surface can interact with the bone grafting material.

Fig. 2 Bone grafting materials with their packaging. Bio-Oss on the left, and Gen-Os porcine on the right (Gen-Os equine presents the same macroscopic product).

Table 1 Overview of used bone grafting materials (manufacturers' data)

Material/Reference	Manufacturer	Origin	Particle granulometry (µm)
Bio-Oss/30643.3	Geistlich	Bovine	250–1,000
Gen-Os/M1005FE	Tecnoss	Equine	250–1,000
Gen-Os/M1005FS	Tecnoss	Porcine	250–1,000

(International Centre for Diffraction Data, Newtown Square, PA, USA). FT-IR spectroscopy was performed on the powdered specimens by preparing KBr discs made up of two to five milligrams of test material ground with 100 mg potassium bromide forming a pellet and tested by FT-IR spectroscope (IRAffinity-1, Shimadzu, Kyoto, Japan) over the range 400–4,000 cm^{-1}.

Powder granulometry
Particle size distribution was performed using laser particle sizer (Hydro 2000, Malvern Mastersizer, Malvern, Worcestershire, UK) using water dispersant. Ten determinations per material were performed. Graphs of volume percent were plotted against the mean diameter in microns.

Cell culture and bone grafting material extract preparation
Human PDL cells were prepared from sound third molars freshly extracted for orthodontics reasons in compliance with French legislation. The extirpated dental ligament was minced, and explants were cultured in 100-mm-diameter culture dishes. Confluent cultures were subcultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 UI/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Dominique Dutscher, Brumath, France). Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in Endothelial Cell Basal Medium 2 (EBM-2) medium with SupplementMix (PromoCell, Heidelberg, Germany). Extracts of each bone grafting material (Table 1, Fig. 2) were prepared by incubating them in serum-free MEM, at 20 mg/mL, for 24 h at 37°C.

Effect of bone grafting materials on VEGF secretion by PDL cells
Conditioned media were prepared by cultivating PDL cells at 30,000 cells/cm^2 in 6-well plates, in 1 mL of the extracts. After 24 h or 3 days, the supernatants (=conditioned media) were collected for VEGF quantification using the DuoSet ELISA kit according to the manufacturer's instructions (R&D Sytems, Lille, France). Supernatants from cells cultured in the same medium but without any prior contact with the materials were used as control.

Effect of bone grafting materials on HUVEC proliferation and neo-angiogenesis
For the assessment of HUVEC proliferation and angiogenesis, conditioned media were prepared according to the same procedure as described above, in serum-free and growth factor free EBM-2 medium, and for a contact period of 3 days with the PDL cell cultures. For evaluation of HUVEC proliferation, the cells were cultured at a low density (1,000 cells/cm^2) in 96-well culture plates. Conditioned media were added onto the cells and proliferation was evaluated after 2, 5 and 7 days using the succinyl dehydrogenase assay (MTT test) as previously described[15]. MTT results are expressed as

percentage cellular viability of the cells cultured in the control medium.
For the assessment of the angiogenic capacity, HUVEC (4×10^5 cells/well) were seeded on Matrigel extracellular matrix and cultured in the conditioned media. The endothelial cell organization was observed with a phase-contrast microscope (Carl Zeiss Axiovert200) (Carl Zeiss S.A.S., Marly Le Roi, France) over a three days period. Angiogenic capacity was quantitatively evaluated using ImageJ software[16] by measuring the tubular perimeters.

Statistics
All biological experiments were repeated at least three times, and statistical significance ($p<0.05$) was determined using the Student t-test to compare the different treatments and their respective controls. Data are expressed as means±standard deviation.

RESULTS

Material characterization
The secondary electron scanning electron micrographs of the three bone grafting materials are seen in Fig. 3. The low power images gave an indication of the particle sizes and shapes of the materials while the higher power images demonstrated the surface morphology. The particle sizes and shapes of the materials varied with the Gen-Os variants having smaller particles sizes and were not as angular when compared to Bio-Oss. The surface textures of the different materials also varied.

All materials were composed of calcium and phosphorus with traces of sodium and magnesium, as calculated from the EDS analyses. Bio-Oss had a lower calcium to phosphate ratio when compared to both equine and porcine Gen-Os (Table 2).

The XRD scans and FT-IR plots are shown in Figs. 4a) and b) respectively. All the bone substitutes exhibited the same phase as indicated by the XRD scans (Fig. 4a). They were poorly crystalline and exhibited the main peaks at 25.88°2θ (0,0,2), a wide peak in the region of 31.31 (2,1,1), 32.18 (3,0,0) and 33.81°2θ (2,0,2) and another minor peak at 39.76°2θ (1,3,0). Other minor peaks, which were also indexed, were present as shown in Fig. 4a). These peaks are typical of calcium oxide phosphate (ICDD: 04-011-1880).

The FT-IR plots (Fig. 4b) show a bifid peak at 550 and 600 cm^{-1} and a stretching vibration at 1,038 cm^{-1}. These are typical of PO4^{-3} and were more pronounced in the Bio-Oss than in the Gen-Os variants. Furthermore absorption bands for CO3^{2-} at 1,463 and 1,412 cm^{-1} and at 872 cm^{-1} reflect a higher incorporation of carbonate in the apatite in Bio-Oss but not in the other materials tested. The 1,641 cm^{-1} band corresponds to H$_2$O and has higher resolution in Bio-Oss. Characteristic OH bands at approximately 3,562 cm^{-1} were present in the Bio-Oss spectrum as well. Both the porcine and equine Gen-Os displayed bands at 1,650 and 1,560 cm^{-1} characteristic of the amide C=O stretching vibrations and N-H bending vibrations. Presence of collagen in both Gen-Os samples
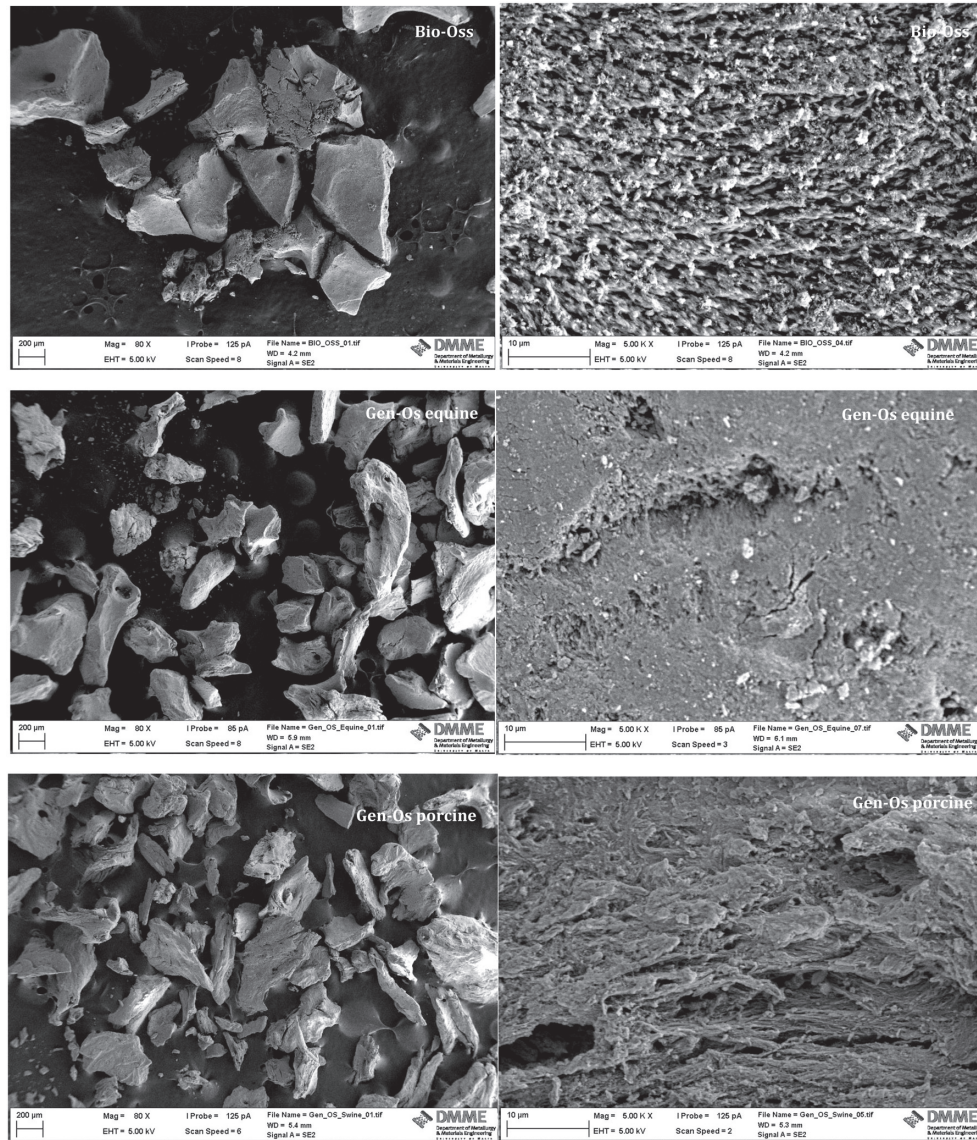
Fig. 3 Secondary electron scanning electron micrographs of the three bone grafting materials at different magnifications showing the surface microstructure of the materials.

Table 2   Calcium to phosphate ratios of test materials

Material	Ca/p ratio
Gen-Os Porcine	2.15
Gen-Os Equine	2.07
Bio-Oss	1.63

also gives bands at 3,450 and 1,450 cm^{-1} from OH$^-$ stretching vibrations.

Powder granulometry
The plots of volume percentage against particle size for the three materials are shown in Fig. 5. The data on particle granulometry as provided by the manufacturer is listed in Table 1. Bio-Oss showed a larger particle size, which verifies the scanning electron microscopy measurements. All the materials exhibited an average particle size distribution between 100–275 µm. The Gen-Os variants had bimodal particle size distribution with particles in the range of 20–80 µm also present.

Effect of bone grafting materials on VEGF secretion by PDL cells
All three bone grafting materials significantly increased VEGF secretion by PDL cells after a contact period of 1 and 3 days (Fig. 6). Compared to basal VEGF secretion (755 pg/mL), this increase was about two-fold higher with
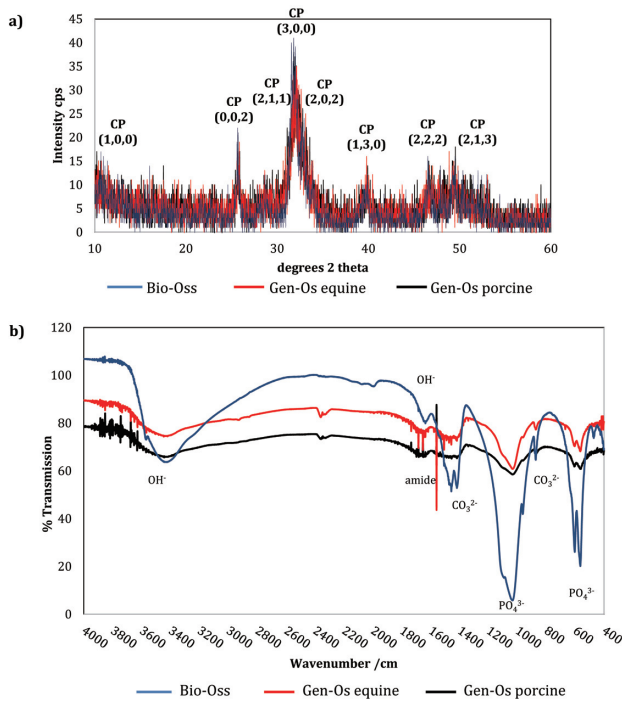
a)

b)

Fig. 4   Material characterization features shown by: (a) X-ray diffraction analysis showing all crystalline phases present. Major peaks belong to calcium oxide phosphate (CP⁻; ICDD: 04-011-1880). (b) Fourier transform infrared (FT-IR) spectroscopy scans showing the main chemical phases present in the materials.
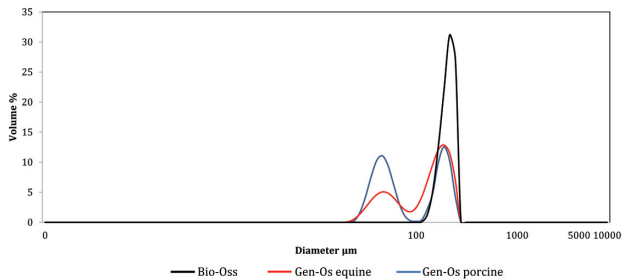

Fig. 5   Particle size distribution measured by laser particle sizer of the three bone grafting materials.
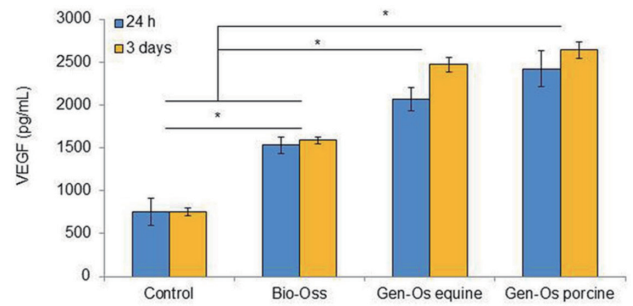

Fig. 6   Effect of bone grafting materials on VEGF secretion.
Quantification of VEGF in conditioned media obtained after incubating extracts from the three bone grafting materials with PDL cells for 24 h and 3 days. PDL cells in serum-free MEM were used as control. Results are expressed as mean±standard deviation. Asterisk corresponds to $p < 0.05$ (Student *t*-test).
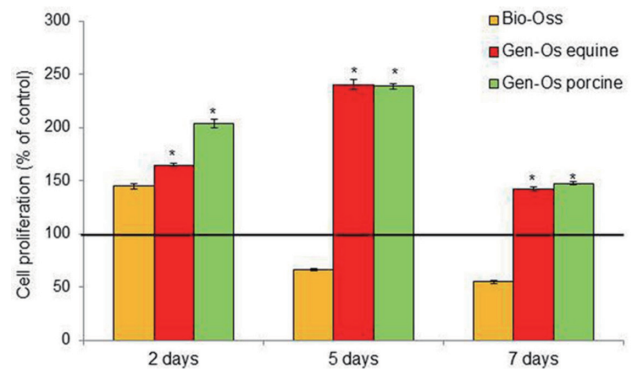

Fig. 7   Effect of bone grafting materials on endothelial cell proliferation.
Proliferation of HUVEC cultures exposed to conditioned EBM-2 medium obtained from three-day PDL cell cultures. Data are obtained after 2, 5 and 7 days and expressed as percentage viability of the control condition (PDL cells in serum-free EBM-2 medium for three days). Results are expressed as mean±standard deviation. All results had statistically significant differences as compared to control condition ($p < 0.05$). Asterisk corresponds to statistically significant differences between both Gen-Os materials and Bio-Oss ($p < 0.05$).

Bio-Oss and about three-fold higher with Gen-Os from porcine and equine origins. Moreover, VEGF secretion was significantly higher with Gen-Os from porcine and equine origins as compared to Bio-Oss.

Effect of bone grafting materials on HUVEC proliferation and neo-angiogenesis
Conditioned media, obtained after a contact period of 3 days with Gen-Os from porcine and equine origins, significantly enhanced endothelial cell proliferation after

2, 5 and 7 days (Fig. 7). A decline in proliferation was noted after 7 days compared to 2 and 5 days, which can be explained by the cells reaching confluency, decreasing their metabolism and thus the rate of MTT reduction to produce formazan[17]. Bio-Oss conditioned medium stimulated endothelial cell proliferation significantly after 2 days, but this effect was abolished after 5 and 7 days of contact where cell viability dropped significantly
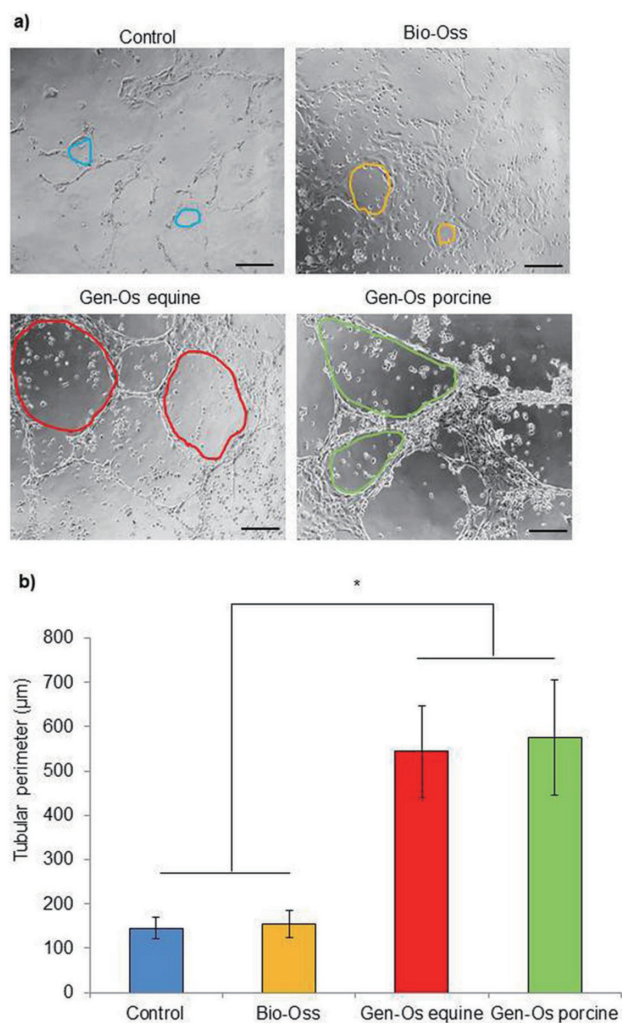
Fig. 8 Effect of bone grafting materials on neo-angiogenesis *in vitro*.
When endothelial cells were incubated in conditioned EBM-2 media obtained from three-day PDL cell cultures, they organized into closed structures and formed a capillary-like network. Conditioned medium obtained from PDL cells exposed to serum-free EBM-2 medium for three days was used as control. (a) Representative microscopic images for perimeter quantification in ImageJ software (scale bar=50 μm). (b) Bar graph of the mean tubular perimeters with error bars presenting standard deviation. Asterisk corresponds to a *p*<0.05.

to 66 and 55%, respectively, of the control condition.

After 3 days of culture on Matrigel, the formation of capillary-like closed structures of well-organized endothelial cells was observed with all bone grafting materials and control (Fig. 8). Measurement of the tubular perimeters demonstrated that conditioned media with Gen-Os from porcine and equine origin significantly enhanced the perimeter of the closed structures as compared to the control and Bio-Oss. The tubular perimeters with Bio-Oss were similar to the control.

DISCUSSION

Three bone grafting materials commonly used in the treatment of periodontal bone defects were evaluated *in vitro* for their angiogenic potential when applied on PDL cells. The rational beyond this work is that some clinical applications of bone grafting materials involve an intimate contact between the material and the periodontal ligament (Fig. 1), and it is hypothesized that this contact may enhance angiogenesis which is essential for the bone regeneration process. Angiogenesis, which is the sprouting of new blood vessels from existing ones, involves endothelial cell stimulation by multiple angiogenic factors such as VEGF, proliferation of endothelial cells and structural rearrangement of these cells to form new well-organized blood vessels[18].

The materials were characterized using a combination of techniques namely SEM and EDS, which gave an indication of particle sizing and shapes. The EDS analysis revealed the elements present within the materials and their ratios. The XRD analysis showed the material crystallinity and the phases present while FT-IR spectroscopy showed the different chemistry. In this way, the precise material chemistry was determined which contributes to the interpretation of the materials' effects on cellular behavior.

Our results clearly indicate a significant increase in VEGF secretion and a higher angiogenic potential of both Gen-Os materials from porcine and equine origins, compared to Bio-Oss. Indeed, VEGF secretion by PDL cells increased when cultured in Gen-Os conditioned media. This was associated with increased endothelial cell proliferation and formation of capillary-like structures. The formation of these structures reflects angiogenesis *in vivo*. Indeed, angiogenic capacity can be evaluated *in vitro* by the Matrigel differentiation assay[19]. With this assay, the formation of capillary-like structures by endothelial cells in response to the biomaterials can be quantitated by counting the number of tubular structures, branching and measurement of tubular perimeters. In this study, tubular perimeters are used as measure of angiogenic potential[20,21] together with assessment of endothelial cell proliferation.

Bio-Oss conditioned medium, on the other hand, induced lower VEGF secretion and less capillary-like structure formation. FT-IR spectroscopy revealed that Bio-Oss exhibits higher incorporation of carbonate in the apatite. Previous works have shown that hydroxyapatite carbonation was associated with lower VEGF secretion as demonstrated with osteoblast-like MG63 cells exposed to experimental carbonated hydroxyapatite compared to pure hydroxyapatite[22]. Furthermore, a possible toxic effect of Bio-Oss was observed in our study since endothelial cell proliferation was significantly decreased after 5 and 7 days of culture. This result is in line with

the study by Zimmerman and coworkers which reported a toxic effect of Bio-Oss eluates on porcine mesenchymal stem cells likely due to toxic substances eluted from the Bio-Oss material during extract preparation[23].

The clinical effectiveness of Bio-Oss for periodontal intrabony defect filling and post extraction ridge preservation has been demonstrated[24], but it should be noted that various studies utilized Bio-Oss Collagen[25,26], as opposed to Bio-Oss without collagen used in this study. For both Gen-Os materials, clinical success has been obtained as well[27,28], and seems to be related to the presence of collagen. The unique manufacturing procedure of Gen-Os materials at low temperature allows the preservation of the natural collagen matrix[29]. The presence of collagen in both Gen-Os materials was confirmed by FT-IR spectroscopy. It has been suggested that the presence of collagen creates a favorable environment for bone regeneration by promoting vascular ingrowth after direct contact between endothelial cells and collagen of the grafting material[30]. Indeed, the specific interaction between integrin and the collagenous amino acid sequence 496–507 on the α1(I) chain, allows direct endothelial cell attachment on the material[31].

The results obtained in this work are based on indirect contact between cell cultures and bone grafting materials. Indeed, extracts prepared from the bone grafting materials were utilized to understand the consequences of the application of a bone grafting material on PDL cells *in vivo*. This work allows illustrating the early molecular and cellular mechanisms underlying the effects observed in clinical studies. As such, this study on the angiogenic potential provides an additional partial explanation for the clinical success observed with Gen-Os materials. Indeed, enhanced VEGF secretion by PDL cells will promote rapid vascularization, which is essential for successful regeneration of the bone tissue in the grafted periodontal defect. Although it remains a short-term *in vitro* study which cannot fully explain the long-term clinical success of these bone grafting materials, our findings provide an interesting lead to be further explored on the *in vivo* level.

CONCLUSION

Within the limits of this *in vitro* study, it was demonstrated that both Gen-Os materials have a higher angiogenic potential compared to Bio-Oss. Both Gen-Os materials have the ability to induce VEGF secretion by PDL cells which is an interesting feature since it mitigates the need for exogenous growth factor delivery. Furthermore, angiogenesis was induced which suggests that the use of Gen-Os materials will favor the bone regeneration process by stimulating early revascularization within the grafted material.

ACKNOWLEDGMENTS

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